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## Activation of Human T Cells Obtained Pre- and Post-Interleukin-2 (IL-2) Therapy by Anti-CD3 Monoclonal Antibody plus IL-2: Implications for Combined In Vivo Treatment

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**Summary:** The role of activated T cells in the mediation of antitumor responses has been documented in several experimental models. In some of these, interleukin-2 (IL-2) has been used as a means to induce and expand the antitumor effects of the T cells. IL-2 has been tested in clinical trials for cancer treatment. Surprisingly, T cells appear to be inactivated by IL-2 in these clinical trials. T cells obtained from peripheral blood after IL-2 therapy showed decreased responses to mitogens and alloantigens, did not proliferate in vitro in response to IL-2, and did not mediate non-major histocompatibility complex-restricted cytotoxicity or targeted lysis in the presence of bispecific monoclonal antibodies. In this study, we present evidence that these post-IL-2 therapy T cells are not irreversibly inactivated; they can be activated in vitro by anti-CD3 monoclonal antibody together with IL-2 to upregulate the p55 component of the IL-2 receptor and proliferate. Nevertheless, following activation by anti-CD3 and IL-2, the level of targeted T-cell cytotoxicity mediated by the post-IL-2 therapy T cells was significantly lower than that by pre-IL-2 therapy T cells. Although in vivo treatment with IL-2 alone induces natural killer (NK) cells to mediate lymphokine-activated killer activity, these data suggest that the T-cell lytic function is inhibited by this treatment and only partially reversible by subsequent T-cell receptor activation using anti-CD3 mAb. Exposure of T cells to anti-CD3 mAb prior to in vivo IL-2 treatment generates T-cell lytic activity in vitro. These results, together with preclinical murine studies, suggest that a combined in vivo protocol of anti-CD3 mAb and IL-2, starting first with the anti-CD3 mAb, may cause activation of the T cells in addition to the activation of NK cells and thus warrant clinical testing. **Key Words:** Activated T cells—Interleukin-2—Anti-CD3 monoclonal antibody—Natural killer cells—Lymphokine-activated killer cells—Peripheral blood lymphocytes.

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Attempts to activate the cellular immune system as clinical immunotherapy for cancer have focused on activation of the natural killer cell (NK) and T-cell effector populations. Numerous murine models document the antitumor efficacy of tumor-reactive

T cells (1-3). Immunotherapy with tumor-infiltrating lymphocytes (TILs) seems to be more efficient than immunotherapy with interleukin-2 (IL-2) infusions alone or combined with in vitro activated lymphokine-activated killer (LAK) cells in some murine tumor models (2,3). Evidence that human T cells may mediate antitumor effects is increasing, particularly for patients with melanoma (4-6). Prior to and after immunotherapy with IL-2 and in vitro expanded heterogeneous populations of TILs, T cells rather than NK cells are predominantly located at the tumor site (4,5).

Clinical testing with TIL cells plus IL-2 has resulted in some antitumor responses but it remains unclear whether this approach might prove to be superior to IL-2 or IL-2 + LAK regimens (7-13). Although LAK activity mediated by CD56+ and CD16+ activated NK cells was induced by in vivo IL-2 therapy (14-17), the rate of antitumor responses resulting in partial or complete tumor regression was relatively low (9,12,13). This limited efficacy of IL-2 therapy may be due to the poor penetration of activated NK cells into the tumor site and may also relate to the inability of IL-2 to activate endogenous T cells in vivo. Circulating T cells obtained from patients following IL-2 therapy did not proliferate in response to further IL-2 in vitro (17,18) and did not lyse tumor targets in a non-major histocompatibility complex (MHC)-restricted manner (14,15,17,18). These T cells also demonstrated decreased responses to mitogens and alloantigens (19).

This present study explores the stimulation of these "defective" T cells with anti-CD3 monoclonal antibody (mAb) and IL-2. Anti-CD3 mAbs are directed against the CD3 antigen, which is associated with the T-cell receptor complex (TCR) (20) and have been shown to have a potent mitogenic effect causing activation and proliferation of T cells (21-25). Following triggering of the CD3/TCR complex by anti-CD3 mAbs, activated T cells show an increased ability to mediate lysis of tumor cells in the presence of bispecific antibodies consisting of an anti-CD3 mAb covalently cross-linked to a mAb directed against a tumor cell antigen (24,25). Bispecific antibodies that bind target cells directly to the CD3 antigen on cytotoxic T cells and induce target cell lysis can be used to monitor the cytolytic activity of T cells (25-29). Activation of T cells through the CD3/TCR complex requires multimeric antigen receptor triggering that can be achieved by triggering the CD3/TCR with immobilized anti-CD3

mAbs (30). Induction of cell proliferation requires an additional signal such as interleukin-1 (IL-1) to enable optimal secretion of IL-2 (23,30). Both requirements can be fulfilled by the use of immobilized anti-CD3 mAbs combined with IL-2.

Peripheral blood T cells from healthy individuals activated in vitro for 5 days with immobilized anti-CD3 and IL-2 were induced to proliferate and to mediate targeted lysis in the presence of bispecific antibodies (29). Therefore, we attempted to activate post-IL-2 therapy T cells in vitro with immobilized anti-CD3 and IL-2. Their activity was compared to the activity generated by similar stimulation of T cells obtained from these same patients prior to in vivo IL-2 therapy. These in vitro studies show that T cells obtained from cancer patients following IL-2 therapy can be stimulated by anti-CD3 and IL-2 to proliferate extensively and to mediate targetable T-cell cytotoxicity but at a lower degree than pre-IL-2 therapy T cells. These data further document that some T-cell functions are impaired by in vivo IL-2 therapy and suggest that initial treatment with anti-CD3 mAb followed by IL-2 may circumvent IL-2-induced T-cell inactivation in cancer patients.

## MATERIALS AND METHODS

### Patient Peripheral Blood Lymphocytes (PBLs)

PBLs from nine cancer patients (designated patients no. 1-9 for these in vitro studies) with renal cell carcinoma or melanoma, participating in clinical IL-2 trials at the University of Wisconsin, were tested in these in vitro studies. IL-2 at a dose of  $3 \times 10^6$  units/m<sup>2</sup>/day was administered weekly for 4 weeks to the patients as a continuous i.v. infusion for 4 days followed by 3 days of observation (11-13). Immunologic and antitumor effects of these treatment regimens, as well as their toxicity and tolerance, have been reported previously (11-13). All patients treated in this protocol signed consent forms for in vivo and in vitro studies approved by the University of Wisconsin Committee for the Protection of Human Subjects. Patients' PBLs were obtained before IL-2 therapy and 24 h after 2- or 4-week cycles of IL-2 therapy (day 13 or day 27) and were separated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. The cells were cryopreserved by controlled rate freezing in 10% dimethyl sulfoxide, stored in liquid N<sub>2</sub>, and thawed on the day of the experiment. Surface phenotype for the markers analyzed was not

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affected by the freezing procedure and LAK activity was retained by these samples (17,31). As controls, PBLs from four healthy normal donors were also tested in this study.

## IL-2

Recombinant human IL-2 for both in vivo and in vitro testing was provided by Hoffmann La Roche, Inc. (Nutley, NJ, U.S.A.). All IL-2 units listed are based on the NCI-BRMP standard IL-2 unit determination.

## Culture Conditions

Following Ficoll-Hypaque separation, PBLs were resuspended at a concentration of  $1 \times 10^6/\text{ml}$  in RPMI 1640 supplemented with 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) buffer, L-glutamine, penicillin/streptomycin, and 10% heat-inactivated pooled nontransfused male serum (Pel-Freez Biologicals, Rogers, AR, U.S.A.) (HS-RPMI). Cells were cultured in HS-RPMI containing either (a) 30 units/ml of IL-2 or (b) anti-CD3 antibody bound to the inner surface of the flask, or (c) immobilized anti-CD3 antibody and 30 units/ml of IL-2. Tissue culture flasks (Costar, Cambridge, MA, U.S.A.) or microwells were precoated with purified anti-CD3 mAb (OKT3 from Ortho Diagnostics, Raritan, NJ, U.S.A.) at 10  $\mu\text{g}/\text{ml}$  in phosphate-buffered saline (PBS) for 1 h at 37°C and washed twice with PBS prior to use. Cells were cultured for 3 days, and then tested in proliferative and cytotoxic assays and their phenotype was determined.

## Cell Depletion

Patient PBLs were depleted of CD56+ and CD16+ NK cells by complement-mediated lysis to recover an enriched T-cell population. Cells were incubated with both the anti-CD56 mAb (the IgM NKH1A from Coulter Immunology, Hialeah, FL, U.S.A.) and the anti-CD16 mAb (the IgM leu11b from Becton Dickinson, Mountain View, CA, U.S.A.) and then with rabbit complement as described previously (17). The effectiveness of the separation was assessed by immunofluorescence (see below). Following depletion, cells were resuspended in HS-RPMI and then assayed or cultured.

## Immunofluorescence for Phenotypic Markers

Cells were washed with PBS and labeled with fluorescent mAbs for 30 min at 4°C, in a standard direct immunofluorescence assay. Cells were washed twice and the lymphocyte fraction was analyzed on a FACScan flow cytometer (Becton Dickinson). All conjugated antibodies were purchased from Becton Dickinson: anti-TAC-fluorescein isothiocyanate (FITC) (which reacts with the p55 IL-2 receptor), anti-leu19 conjugated to phycoerythrin (PE) (CD56, which reacts with a 220 kDa protein expressed on NK cells), anti-leu4-PE (directed against the CD3 component of the T-cell receptor complex on the T cells), and anti-CD4-PE and anti-CD8-PE (which react with the T helper/inducer cells and the T cytotoxic/suppressor cells).

## Cell-Mediated Lysis (CML) Assay

Cells were seeded in 96-well U-bottomed microtiter plates in HS-RPMI and assayed in the presence of anti-CD3 (0.25  $\mu\text{g}/\text{ml}$ ) or 100 ng/ml of a bispecific antibody anti-CD3-113F1. The bispecific antibody CD3-113F1 was prepared as described previously (26,27) and consists of anti-CD3 mAb directed against the CD3 determinant on the T cells (OKT3 from Ortho Diagnostics) conjugated to mAb 113F1 directed against colon carcinoma cells (generously provided by Dr. David Ring, Cetus Corporation, Emeryville, CA, U.S.A.). The NK-resistant LS 174T human colon carcinoma cell line (American Tissue Type Collection, Rockville, MD, U.S.A.) recognized by mAb 113F1 was used as target cells and labeled with  $^{51}\text{Cr}$ , and then added to the cells as described previously (17). Following incubation for 4 h at 37°C, the plates were processed, and the percent cytotoxicity was calculated using the mean of triplicate wells and converted into lytic units (LU) as described previously (17,31). One lytic unit was defined as the number of effector cells causing 20% lysis of  $5 \times 10^3$  target cells; lytic units are expressed as LU/ $10^7$  effector cells.

## Proliferative Assay

Cells at  $1 \times 10^5$  cells/well in 0.2 ml of HS-RPMI were incubated in U-bottomed microplates in medium alone or in the presence of 30 or 100 units/ml of IL-2 or in anti-CD3-coated wells in the absence or presence of IL-2 at 30 or 100 units/ml. After 3 days at 37°C in 5%  $\text{CO}_2$ , cells were pulse labeled

with 1  $\mu$ Ci of [ $^3$ H]thymidine ([ $^3$ H]TdR) (New England Nuclear, Boston, MA, U.S.A.) for 18 h. Cultures were harvested with a Mash harvester (Otto Hiller, Madison, WI, U.S.A.) and counted by liquid scintillation. The mean cpm of quadruplicate or triplicate samples are reported.

## RESULTS

### Proliferation of PBLs Activated with CD3 + IL-2

Both the NK- and T-cell populations present in resting PBLs obtained from healthy individuals or cancer patients before IL-2 therapy are induced to proliferate in response to IL-2 in vitro (17). However, after IL-2 therapy, the response of PBLs to IL-2 in vitro was primarily mediated by NK cells while T cells showed virtually no responsiveness to this stimulant (17,18). These T cells showed reduced responsiveness to other stimuli such as the mitogen phytohemagglutinin or allogeneic PBLs (19). The activation of these "unresponsive" T cells through the CD3/TCR complex using immobilized anti-CD3 mAb was tested in this study. Preliminary experiments showed that the proliferative response of PBLs to anti-CD3 mAb combined with 100 units/ml of IL-2 increased after 1-3 days in culture and within 3 days was considerably greater than the proliferation induced by anti-CD3 alone or IL-2 alone (data not shown). This confirms the synergistic interaction between anti-CD3 mAb and IL-2 previously described (32). Since the steady-state concentration of IL-2 measured in the serum of these patients treated with our IL-2 protocol was approximately 30 units/ml (33), the proliferative response of PBL obtained before and after 4 weeks of in vivo IL-2 therapy was tested following 3 days of culture in immobilized anti-CD3 mAb in the presence or absence of 30 or 100 units/ml of IL-2.

Data from a representative patient (no. 1) are shown in Table 1. The proliferative response of resting pretherapy PBLs to anti-CD3 + IL-2 was higher than the response to anti-CD3 alone and >10-fold greater than the response to 100 or 30 units/ml of IL-2 alone. The magnitude of this proliferative response to anti-CD3 + IL-2 was comparable to the response mediated by normal resting PBLs from healthy individuals (data not shown). Posttherapy PBLs showed a greater proliferative response to IL-2 than did pretherapy PBLs (Table 1), as shown previously (19). These posttherapy PBLs also showed a greater response to anti-CD3

TABLE 1. Proliferative response of pre- and post-IL-2 therapy PBLs to IL-2, anti-CD3 mAb, or anti-CD3 + IL-2

Stimuli	[ $^3$ H]TdR incorporation (cpm)	
	Pre-IL-2 therapy	Post-IL-2 therapy
Medium	742 $\pm$ 102	511 $\pm$ 274
IL-2 (30)	3,142 $\pm$ 127	12,991 $\pm$ 145
IL-2 (100)	5,161 $\pm$ 351	27,398 $\pm$ 1,087
CD3	71,962 $\pm$ 10,437	21,274 $\pm$ 3,722
CD3 + IL-2 (30)	91,179 $\pm$ 3,242	41,380 $\pm$ 1,645
CD3 + IL-2 (100)	87,169 $\pm$ 6,372	41,273 $\pm$ 1,404

PBLs obtained from cancer patient no. 1 before or after 4-week cycles of IL-2 therapy were incubated at  $1 \times 10^5$  cells/well in medium, or in IL-2 at 30 or 100 units/ml, or in anti-CD3-coated wells in the absence or presence of IL-2 at 30 or 100 units/ml. After 3 days, plates were labeled with [ $^3$ H]thymidine for 18 h and harvested. Mean cpm of triplicates  $\pm$  SD are reported.

mAb combined with IL-2 than to IL-2 alone at 30 or 100 units/ml, indicating that the T cells might respond to activation by anti-CD3 (Table 1). Unlike the dose-dependent response to IL-2 alone, both 30 and 100 units/ml of IL-2 induced comparable proliferative responses when combined with anti-CD3 mAb; this was noted for pre- and post-IL-2 therapy PBLs. Although posttherapy PBLs proliferate strongly to anti-CD3 + IL-2, this response remains less than that of pretherapy PBLs to these same stimuli (Table 1).

Because anti-CD3 mAb activates T cells via the TCR, this difference between pre- and posttherapy PBLs proliferative responses to anti-CD3 + IL-2 may reflect the difference in the T-cell composition of these two samples (Table 2). PBLs obtained before therapy from patient no. 1 consisted of 80% CD3+ T cells and 14% CD56+ NK cells, similar to resting PBLs from control donors. In contrast, PBLs obtained from the same patient no. 1 following IL-2 therapy showed a considerable expansion of CD56+ NK cells (80%) at the expense of the CD3+ T cells (22%) (Table 2), as described previously (17). The decrease in CD3+ T cells (22%) is reflected in the decrease in the percent of CD4+ T cells (19%) while the percent CD8+ cells (48%) increased. Two color analysis revealed that only 8% CD8+ cells coexpressed the CD3 antigen and were T cells while 36% CD8+ cells coexpressed CD56 antigen and thus were of the NK phenotype (data not shown). These data confirm prior analyses demonstrating the expression of CD8 by activated CD56+ NK cells obtained following IL-2 therapy (16). Further culture of posttherapy PBLs in vitro in

TABLE 2. Upregulation of TAC antigen on PBLs stimulated with anti-CD3 and IL-2

PBL sample	Cultured in	% Cells positive for markers							
		Single marker analysis					Two-color analysis <sup>a</sup>		
		CD56	CD3	CD4	CD8	TAC	TAC/CD3	TAC/CD4	TAC/CD8
Pre-IL-2 therapy	No culture	14	80	64	14	6	5	5	1
	IL-2	8	84	70	16	12	8	8	0
	CD3 + IL-2	10	95	89	16	93	92	85	12
Post-IL-2 therapy	No culture	80	22	19	48	9	7	7	1
	IL-2	75	27	24	50	13	7	7	1
	CD3 + IL-2	50	54	47	47	61	58	50	9

PBLs obtained from cancer patient no. 1 before and after in vivo IL-2 therapy were analyzed for surface markers by flow cytometry before in vitro culture or after 3 days of culture with 30 units/ml of IL-2 alone or combined with immobilized anti-CD3 mAb. Cells were labeled with anti-CD56-PE, anti-CD3-PE, anti-CD4-PE, anti-CD8-PE, or anti-TAC-FITC.

<sup>a</sup> For two-color analysis, the cells were labeled with anti-TAC-FITC combined with anti-CD3-PE, anti-CD4-PE, or anti-CD8-PE. Gates were set for nonspecific binding using cells labeled with mouse IgG<sub>1</sub>-PE and mouse IgG<sub>1</sub>-FITC.

30 units/ml of IL-2 for 3 days did not change the surface marker phenotype of these cells while activation by anti-CD3 + IL-2 caused an expansion of T cells (Table 2). The percent T cells in posttherapy PBLs increased from 22 to 54%, probably resulting from the proliferative response of T cells to anti-CD3 stimulation (Table 2). Virtually all of these posttherapy T cells stimulated by anti-CD3 + IL-2 expressed TAC on their surface with a high intensity of staining on all of the CD4+ T cells but only 9% of the CD8+ cells, which are probably the CD8+/CD3+ T cells described above (Table 2). The induction by anti-CD3 + IL-2 of TAC upregulation on T cells from post-IL-2 therapy PBLs indicates that these posttherapy T cells are responsive to activation in vitro by anti-CD3 and are probably induced to proliferate (Table 1). Upregulation of TAC on posttherapy T cells was not seen following culture in IL-2 alone (Table 2), confirming our previous findings (18).

T cells obtained before IL-2 therapy also upregulated TAC following 3 days of in vitro activation with anti-CD3 + IL-2 (Table 2). Before activation, TAC was expressed by a small fraction of the CD4+ T cells while CD8+ T cells did not express TAC. Following activation with anti-CD3 + IL-2, the majority of CD4+ T cells and CD8+ T cells express TAC, showing a high intensity of staining indicating a high density of this IL-2 receptor on these cells. This IL-2 receptor upregulation on the majority of the pretherapy PBLs consisting predominantly of CD3+ T cells (95%) may account for the greater proliferative response of these PBLs to anti-CD3 + IL-2 than the response by posttherapy PBLs consisting of 54% T cells (Tables 1 and 2).

In several experiments, the phenotype of the cells activated by anti-CD3 alone was similar to that obtained by anti-CD3 + IL-2 activation and was characterized by expression of TAC on most T cells (data not shown).

Comparable in vitro data were obtained with PBLs from four other cancer patients and PBLs from two healthy individuals showed responses similar to those by the pretherapy patient PBLs.

#### Proliferative Response of T Cells Separated from Pre- and Post-IL-2 Therapy PBLs

We have shown previously that the proliferative response of post-IL-2 therapy PBLs to in vitro IL-2 is mediated predominantly by activated NK cells (17,18); the data summarized in Tables 1 and 2 indicate that this response can be augmented by exposing the cells to anti-CD3 mAb. Stimulation with anti-CD3 mAb, acting at the level of the CD3/TCR complex of the T cells, appears to have induced the posttherapy T cells to proliferate. To test this hypothesis, T cells were separated from PBLs obtained from patient no. 2 following 2-week cycles of IL-2 therapy, and compared to the response by resting T cells separated from PBLs obtained from this same patient before IL-2 therapy began (Table 3). As previously shown (17,18), the T cells isolated from posttherapy PBLs showed a minimal proliferative response to IL-2 at 30 units/ml [the response remained low even at a higher concentration of IL-2, including 100 or 400 units/ml (data not shown)]. In contrast to their lack of responsiveness to IL-2, these posttherapy T cells proliferated in response to anti-CD3 (Table 3). This response was considerably

TABLE 3. Anti-CD3-induced proliferation of T cells separated from pre- and posttherapy patient PBLs

PBL sample	[ <sup>3</sup> H]TdR incorporation (cpm)			
	Medium	IL-2	CD3	CD3 + IL-2
Pretherapy				
Unseparated	312 ± 244	1,310 ± 51	27,723 ± 3,519	39,184 ± 6,637
T cells	306 ± 238	1,009 ± 169	29,456 ± 1,834	51,313 ± 2,578
Posttherapy				
Unseparated	195 ± 93	3,439 ± 1,424	51,987 ± 1,952	73,021 ± 3,816
T cells	158 ± 60	345 ± 46	35,225 ± 1,504	126,338 ± 6,866

PBLs obtained from cancer patient no. 2 before and after two cycles of IL-2 therapy were depleted of NK cells by complement-mediated lysis (see the Materials and Methods section). The T cells recovered were 98–99% CD3+. Unseparated and T-cell populations were incubated for 3 days at  $1 \times 10^5$  cells/well in medium alone or with IL-2 (30 units/ml) or in anti-CD3-coated wells in the absence or presence of 30 units/ml of IL-2. Plates were then labeled with [<sup>3</sup>H]thymidine for 18 h and harvested. Mean cpm of quadruplicates (for unseparated samples) or triplicates (for T-cell samples) ± SD are reported.

enhanced by addition of IL-2 and was even greater than that of unseparated PBLs, which reflects the response of both NK and T cells (Table 3). PBLs from patient no. 2 following two cycles of IL-2 therapy consisted of a majority of T cells (70%) and only 30% CD56+ NK cells; therefore, their response to anti-CD3 + IL-2 was greater than the response obtained from PBLs from patient no. 1 following four cycles of IL-2 therapy, which consisted of a lower T-cell population (22%) and a greater NK cell population (80%) (Tables 1 and 2). This change in phenotype that can be seen following 2- and 4-week cycles of IL-2 therapy and between patients illustrates biological variations between patients often noted in clinical trials. Nevertheless despite differences in the percent of T cells present in the PBLs obtained from several patients following IL-2 therapy, these circulating T cells were all poorly responsive to IL-2 but could be induced to proliferate by anti-CD3 + IL-2.

The T cells separated from pretherapy resting PBLs also proliferated intensively to anti-CD3 or anti-CD3 + IL-2 in a 3-day assay while their response to IL-2 was low in this short-term culture (19).

#### Targeted T-Cell Cytotoxicity Mediated by Cells Activated with CD3 and IL-2

Following IL-2 therapy, PBLs have demonstrated IL-2-dependent LAK activity mediated primarily by activated CD56+ NK cells (11,17,31). Circulating lymphocytes obtained 24 h after cessation of IL-2 infusion mediate LAK activity that can be augmented by inclusion of IL-2 in the <sup>51</sup>Cr release assay and considerably boosted by preincuba-

tion of the cells in IL-2 for 24 h prior to the CML assay (31,34). We have shown that T cells were not involved in this LAK cytotoxic activity (17,18). We therefore tested whether these same T cells were able to mediate redirected lysis against tumor cells in the presence of bispecific antibodies. PBLs obtained from patient no. 3 following four cycles of IL-2 therapy were tested for their lytic potential against the tumor cells LS174T in the absence or presence of the bispecific antibodies CD3–113F1. The CD3–113F1 heteroconjugate binds to the effector T cell through the anti-CD3 mAb and binds to the LS174T target through the 113F1 mAb, and thus connects the effector cell with the target cell.

As a control for targeted T-cell lysis, fresh PBLs from a control donor were tested in the same experiment. These resting lymphocytes mediated significant lysis against LS174T only in the presence of CD3–113F1 bispecific mAbs. This targeted lysis was further enhanced after 24 h of incubation in IL-2 or anti-CD3 + IL-2 (Table 4), as previously shown (29). Anti-CD3 alone added to the assay did not increase the amount of cytotoxicity. The targeted lytic activity by unstimulated normal lymphocytes measured in the presence of CD3–113F1 mAbs, which does not require preactivation with anti-CD3, may be mediated by the CD3+/CD56+ T-cell subpopulation, present in low buoyant density percoll fraction (29).

PBLs obtained after IL-2 therapy mediated lysis of LS 174T that was enhanced by inclusion of IL-2 in the assay and this cytolytic activity was dramatically increased after culturing these PBLs for 24 h in IL-2 or anti-CD3 + IL-2 (Table 4). The addition of CD3–113F1 bispecific mAbs failed to augment this IL-2-dependent LAK activity before or after



TABLE 4. Targeted lytic activity of post-IL-2 therapy patient PBLs and normal resting lymphocytes

Donor	mAb added to 4 h assay	Lytic units/10 <sup>7</sup> effectors					
		Day 0		After 24-h culture in <sup>b</sup>			
		Medium <sup>a</sup>	IL-2 <sup>a</sup>	Medium	IL-2	CD3	CD3 + IL-2
Normal PBLs	Medium	48	12	21	69	27	264
	CD3	45	13	0	67	16	274
	CD3-113F1	248	201	401	473	190	472
Post-IL-2 therapy	Medium	108	303	44	1,971	397	1,599
	CD3	67	236	60	1,582	475	1,352
	CD3-113F1	88	223	105	1,335	438	1,403

Fresh PBLs from a healthy individual and PBLs obtained from cancer patient no. 3 after four cycles of IL-2 therapy were assayed for cytotoxicity against <sup>51</sup>Cr-labeled LS 174T target before culture (day 0) or after 24-h culture in medium or IL-2 (100 units/ml) or immobilized anti-CD3 in the absence or presence of IL-2. The 4-h CML assay was done in medium with anti-CD3 or the bispecific antibody CD3-113F1.

<sup>a</sup> Assayed 4 h in CML in medium or IL-2 with medium, CD3, or CD3-113F1.

<sup>b</sup> Assayed 4 h in CML in medium alone with medium, CD3, or CD3-113F1.

culture (Table 4). PBLs from three other patients obtained after IL-2 therapy also did not demonstrate detectable targeted lysis with the CD3-113F1 mAbs. The targeted lysis might not have been detected because the high level of killing mediated by the *in vivo* activated NK cells could have masked this T-cell effect.

#### Targeted Cytotoxic Activity of T Cells Separated from Pre- and Posttherapy Patient PBLs Activated with CD3 and IL-2

We have shown in the previous section (Table 4) that the lytic activity of PBLs obtained after therapy was not enhanced by the bispecific heteroconjugate that contains anti-CD3. Posttherapy T cells might require prior *in vitro* activation through the CD3/TCR to mediate targeted lysis. Previous studies have documented that a short-term activation of T cells (3-5 days) with anti-CD3 induces antitumor cytolytic activity measurable in the presence of bispecific antibodies (24,25,29). This targeted cytotoxicity has been found to be mediated by the CD3+/CD56- T cells that are present in the high buoyant density percoll fraction and require preactivation of the CD3/TCR receptor unlike the CD3+/CD56+ T cell subpopulation (29). To determine whether posttherapy T cells can be induced to mediate targeted lysis following stimulation with anti-CD3 + IL-2 *in vitro*, T cells were separated from PBLs obtained from patient no. 2 following two cycles of IL-2 therapy by selective depletion of CD56+ and CD16+ NK cells and were cultured for

3 days in anti-CD3 + IL-2 or in IL-2 alone. As a control, pretherapy PBLs from the same patient were also depleted of NK cells and the recovered T-cell population was assayed in the same experiment. The proliferative responses of these unseparated and separated T-cell populations obtained following 3 days of incubation in anti-CD3 + IL-2 are detailed in Table 3 (described above). Prior to testing these cultured cells in the cytotoxic assay against the LS174T target, the cells activated with anti-CD3 + IL-2 were washed and cultured for 1 day more in medium alone to reduce the IL-2-dependent activity of NK cells and to wash off residual anti-CD3 mAb, allowing for recovery of the CD3 antigen (29). The cells activated with IL-2 alone were maintained in IL-2 prior to the CML assay to retain strong LAK activity mediated by NK cells, as a positive cytolytic control. Unseparated populations of pretherapy PBLs activated with IL-2 alone showed high levels of lytic activity in the presence or absence of bispecific antibody probably reflecting LAK activity mediated by NK and T cells (Table 5). In contrast, the lytic activity mediated by cells activated with anti-CD3 and IL-2 and then cultured in medium alone was lower than that of cultures maintained in IL-2 but was boosted by inclusion of CD3-113F1 bispecific antibody indicating T-cell activation by anti-CD3. A dramatic augmentation of this targeted cytotoxicity was observed in the T-cell enriched population stimulated by anti-CD3 + IL-2 while a lower level of targeted lysis was observed in T cells activated by IL-2 alone (Table 5). T cells activated by IL-2 alone also dem-



TABLE 5. Lytic ability of T cells separated from pre- and posttherapy patient PBLs following stimulation with anti-CD3 and IL-2

PBL sample	mAb added to 4-h assay	Lytic units/10 <sup>7</sup> effectors cultured in	
		IL-2	CD3 + IL-2
Pretherapy			
Unseparated	Medium	668	78
	CD3-113F1	676	323
T cells	Medium	241	109
	CD3-113F1	375	953
Posttherapy			
Unseparated	Medium	707	165
	CD3-113F1	622	199
T cells	Medium	0	0
	CD3-113F1	0	72

PBLs obtained from cancer patient no. 2 before and after two cycles of IL-2 therapy were depleted of NK cells by complement-mediated lysis. The T cells recovered were 98–99% CD3+. Unseparated and T-cell populations were cultured for 3 days at  $5 \times 10^5$ /ml in 30 units/ml of IL-2 or in immobilized anti-CD3 and 30 units/ml of IL-2. Then the cells were washed, incubated for one more day in medium, and assayed for cytotoxicity, in medium or in the presence of the bispecific antibody CD3-113F1, against <sup>51</sup>Cr-labeled LS 174T target. In several experiments, the mAbs 113F1 and anti-CD3 alone did not increase the lytic activity of the cells.

onstrated some antibody-independent non-MHC-restricted cytotoxic activity against LS174T tumor target although this activity was lower than that of unseparated populations consisting of both NK- and T-cell populations (17).

In contrast to pretherapy T cells, posttherapy T cells cultured in IL-2 alone did not kill LS174T target cells while unseparated PBLs exhibited good LAK activity, likely mediated by activated NK cells, as shown previously (17). This confirms our previous findings that post-IL-2 therapy T cells cannot mediate non-MHC-restricted cytotoxicity (17,18). Although stimulation of these posttherapy T cells from patient no. 2 by anti-CD3 + IL-2 induced a strong proliferative response (as shown in Table 3), these stimulated T cells mediated only a low level of targeted cytolytic activity detectable in the presence of bispecific mAbs (Table 5). This targeted lytic activity is masked by the LAK activity mediated by NK cells in the unseparated posttherapy PBLs. It should be noted that purification of the T cells by double depletion of all NK cells with anti-CD56 and anti-CD16 resulted also in depletion of the small component of CD3+/CD56+ T cells. The targeted lytic ability of these CD3+/CD56+ cells (29), which constitute only 2–5% of the PBLs obtained before or after IL-2 therapy (17), could there-

fore not be assessed separately in this experiment. Nevertheless, unseparated posttherapy PBLs stimulated with anti-CD3 + IL-2 did not show an increased lytic activity in the presence of CD3-113F1, suggesting that the CD3+/CD56+ component might be of minimal significance in the lytic ability of these posttherapy T cells. These data were reproduced with T cells obtained from two other patients.

## DISCUSSION

Following treatment of cancer patients with IL-2, PBLs isolated from these patients demonstrated LAK activity in vitro mediated primarily by activated CD56+ NK cells (17). These CD56+ NK cells could be induced to proliferate to IL-2 in vitro with more rapid kinetics than could resting PBLs (17,19) and they mediated high levels of non-MHC-restricted cytotoxicity in the presence of IL-2 against fresh tumor cells or tumor cell lines (17,31,34). In contrast to the activation of the NK-cell population, in vivo IL-2 therapy seems to impair the functions of the T-cell population. Post-IL-2 therapy T cells isolated from the peripheral blood of the patients showed decreased responses to mitogens and alloantigens (19) and were poorly responsive to IL-2 (17,18). They did not proliferate in response to further exposure to IL-2 in vitro (17,18), possibly as a result of nonfunctional IL-2 receptors (18). These T cells also did not mediate non-MHC-restricted cytotoxicity against tumor targets (17,18). However, T cells obtained before IL-2 therapy are able to generate both a proliferative response and LAK activity after culture in IL-2 (17). The clinical significance of these impaired T-cell functions in cancer patients receiving IL-2 therapy remains uncertain. It should be noted that only the circulating T cells could be assessed in this study. The functional changes of the posttherapy T cells documented in this manuscript and others (17–19) may not reflect changes in the function of the T cells segregated in lymphoid organs or tumor areas that could not be readily evaluated.

This study presents evidence that post-IL-2 therapy T cells are not irreversibly impaired by IL-2 therapy; they can be activated in vitro with immobilized anti-CD3 mAb. This activation through the CD3/TCR complex induced the expression of a high density of the TAC (p55) IL-2 receptor on all T cells including both the CD4+ and CD8+ T-cell populations. The upregulation of TAC also observed following anti-CD3 stimulation of resting pre-IL-2

therapy T cells is indicative of T-cell activation by anti-CD3, as previously documented in vitro (24) and in vivo (35,36). These TAC molecules are probably associated with the p70 chain of the IL-2 receptor in the form of functional high-affinity IL-2 receptor complexes. Wang and Smith have demonstrated that PBLs activated for 3 days with anti-CD3 proliferate in response to IL-2 using high-affinity IL-2 receptor complexes (37). This may account for the rapid and extensive proliferation of both the pre- and post-IL-2 therapy T cells in response to the relatively low concentration of exogenous IL-2 (30 units/ml) that was added to the anti-CD3 mAb in the culture. Both the upregulation of TAC molecules on T cells and the extensive proliferation of the cells were not observed in cells cultured with IL-2 alone for a similar short-term period (3 days).

Several studies have documented that the CD3/TCR complex is involved in the mediation of T-cell cytotoxicity. Following triggering of this receptor by anti-CD3 mAb for 3–5 days, normal T cells have demonstrated antitumor T-cell cytolytic activity measurable in the presence of bispecific antibodies containing anti-CD3 mAb covalently cross-linked to a mAb directed against a tumor cell antigen (24–29). This targeted cytotoxicity has been found to be mediated by the CD3+/CD56– T cells (29). Similar to results with anti-CD3 activated T cells obtained from healthy donors, anti-CD3 + IL-2 activation of pretherapy T cells obtained from cancer patients generated significant targeted cytotoxic activity against the colon carcinoma cell line LS 174T in the presence of the bispecific antibodies consisting of anti-CD3 and 113F1 mAb specific for colon carcinoma cells. Activation by IL-2 alone generated lower but significant levels of targeted lysis. In contrast, posttherapy T cells demonstrated relatively low targetable cytotoxic activity following stimulation with anti-CD3 + IL-2. In addition, before in vitro stimulation with anti-CD3, posttherapy T cells did not mediate LAK activity and targeted lysis, while normal resting T cells did show targeted lysis in the presence of bispecific antibodies, a lytic activity likely to be mediated by the small CD3+/CD56+ T-cell subpopulation (29). Post-IL-2 therapy T cells have also shown decreased MHC-restricted CTL activity (19) and thus may be defective in their lytic machinery. These data indicate that IL-2 therapy affects the function of T cells and anti-CD3 + IL-2 activation of these cells in vitro can only partially restore this function.

While activation of NK cells in vivo with IL-2 therapy appears to induce some clinical antitumor effects, further antitumor activity might be obtained by also activating T-cell function. This was one of the goals and rationales for initiating clinical IL-2 treatment. The studies shown here confirm the defective T-cell function seen in IL-2-treated patients. Before therapy was begun, these same patients showed strong T-cell responses induced in vitro through the TCR complex by the combination of anti-CD3 + IL-2. These in vitro data, together with previous human and murine studies (24,38–41), suggest activation in vivo with anti-CD3 and IL-2 might induce both augmented T-cell and NK function, especially if the anti-CD3 mAb treatment precedes IL-2 treatment. Such a combination of anti-CD3 + IL-2 that might activate both the NK- and T-cell populations could also aid in the design of subsequent immunotherapy protocols potentially including in vivo treatment with anti-CD3 bispecific antibodies that may facilitate localization and tumor-specific destruction by in vivo activated effector cells. Furthermore, T cells activated by anti-CD3 + IL-2 might secrete additional antitumor lymphokines that might contribute to greater tumor destruction. This is suggested by the findings that in vivo administration of anti-CD3 in mice caused tumor necrosis factor secretion (35) and increased levels of GM-CSF in serum leading to induction of hematopoiesis (36) and has induced antitumor responses (35).

Just as the most effective chemotherapies seem to be combinations of independently acting cytotoxic agents, it seems likely that a multimodal immunologic attack would be more effective than a single immune mechanism. Thus, a combined approach involving both NK- and T-cell activation, coupled with infusions of intact mAb to enable conventional antibody-dependent cell killing (42) and infusions of bifunctional mAb to enable "retargeted" killing, would seem to be more effective than NK activation alone with IL-2. Initial steps towards this goal, combining IL-2 with anti-CD3 mAb in a phase I clinical trial, are now being initiated.

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